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Derivs. of ML-236B - which are cholesterol biosynthesis inhibitors useful in treating hypercholesterolaemia

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Number of Countries: 014 Number of Patents: 027

Patent Family:

Patent No	Kind	Date	Applicat No	Kind	Date	Week
X GB 2077264	A	198111216	GB 8117450	A	19810608	198151 B
BE 889150	A	198111209				198152
DE 3122499	A	198111224	DE 3122499	A	19810605	198201
FR 2483912	A	198111211				198203
SE 8103560	A	19820104				198203
NL 8102737	A	19820104				198205
DK 8102470	A	19820111				198206
JP 57002240	A	19820107	JP 8076127	A	19800606	198207
FI 8101762	A	19820129				198209
JP 57050894	A	19820325	JP 80124385	A	19800908	198218
JP 57108039	A	19820705	JP 80130311	A	19800919	198232
JP 57067575	A	19820424	JP 80115483	A	19800822	198235
US 4346227	A	19820824	US 82351975	A	19820224	198236
CA 1150170	A	19830719				198335
AT 8102567	A	19830915				198340
US 4410629	A	19831018				198344
GB 2077264	B	19840426				198417
US 4448979	A	19840515				198422
CH 655090	A	19860327				198617
JP 86013699	B	19860415				198619
DE 3122499	C	19871126				198747
JP 87054476	B	19871116				198749
SE 453389	B	19880201				198807
JP 88021672	B	19880509				198822
IT 1144598	B	19861029				198833
JP 88048858	B	19880930				198843
NL 191738	B	19960102	NL 812737	A	19810605	199607

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GB 2077264	A		28		
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Abstract (Basic): GB 2077264 A

ML-236B derivs. of formula (I) ring-closed lactones, salts and esters are new: where R is a gp. (II) or (III): (I) are cholesterol biosynthesis inhibitors and are thus useful in treating hypercholesterolaemia.

(I) are prepd. by enzymatic hydroxylation of ML-236B, or ML-236B carboxylic acid or its salt or ester (see US398140). Pref. the enzyme is provided by a strain of Mucor, Phizopus, zygorynchus, circinella, Actinomucor, Gongronella, Phyomyces, Mortierella, Pycnoporus, Rhizoctonia, Absidia, Cunninghamella, Syncephalastrum or Streptomyces. A partic. suitable microorganism is Mucor hiemalis F. hiemalis, which gives at least 90% conversion of ML-236B and its derivs.

Title Terms: DERIVATIVE; ML; CHOLESTEROL; BIOSYNTHESIS; INHIBIT; USEFUL; TREAT; HYPERCHOLESTEROLAEMIC

Derwent Class: B03; B05; D16

International Patent Class (Main): C12P-007/62

International Patent Class (Additional): A61K-031/22; A61K-031/365;

C07C-059/90; C07C-069/03; C07C-069/22; C07D-309/30; C12P-017/06;

C12R-001/46
File Segment: CPI
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1. The first part of the document is a list of names and addresses of the persons who were interviewed for the purpose of this study.

2. The second part of the document is a list of the questions which were asked of the interviewees, and the answers which were given to them.

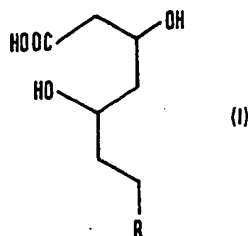


(12) UK Patent Application (19) GB (11) 2 077 264 A

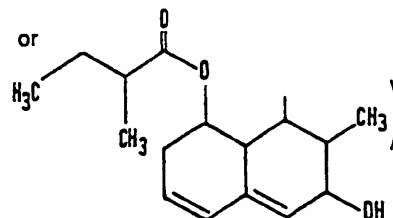
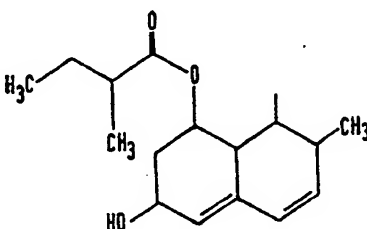
- (21) Application No 8117450
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 55/130311
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 306 30Y 351 352 360 362
 366 367 368 36Y 389 625
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 TV
 (56) Documents cited
 None
 (58) Field of search
 C2C
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(54) ML-236B derivatives and their preparation

(57) Compounds of formula (I):



(wherein R represents a group of formula



and the corresponding ring-closed lactones, salts (especially alkali metal salts) and esters (especially C₁—C₅ alkyl esters) thereof may be prepared by subjecting ML-236B, or ML-236B carboxylic acid or a salt or ester thereof to enzymatic hydroxylation, which may be effected by means of microorganisms of the genera Mucor, Rhizopus, Zygorhynchus, Circinella, Actinomucor, Gongronella, Phycomyces, Martierella, Pycnoporus, Rhizoctonia, Absidia, Cunninghamella, Syncephalosporum and Streptomyces, or cell-free, enzyme-containing extracts from said microorganisms. The compounds are capable of *inhibiting biosynthesis of cholesterol* and are thus useful in the treatment of *hypercholesteremia*.

GB 2 077 264 A

Fig 1

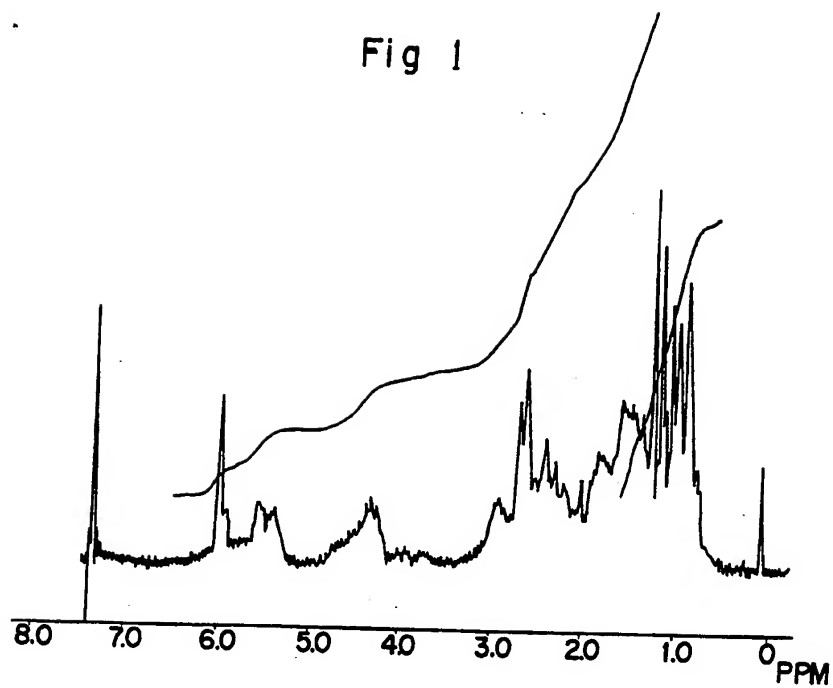


Fig 2

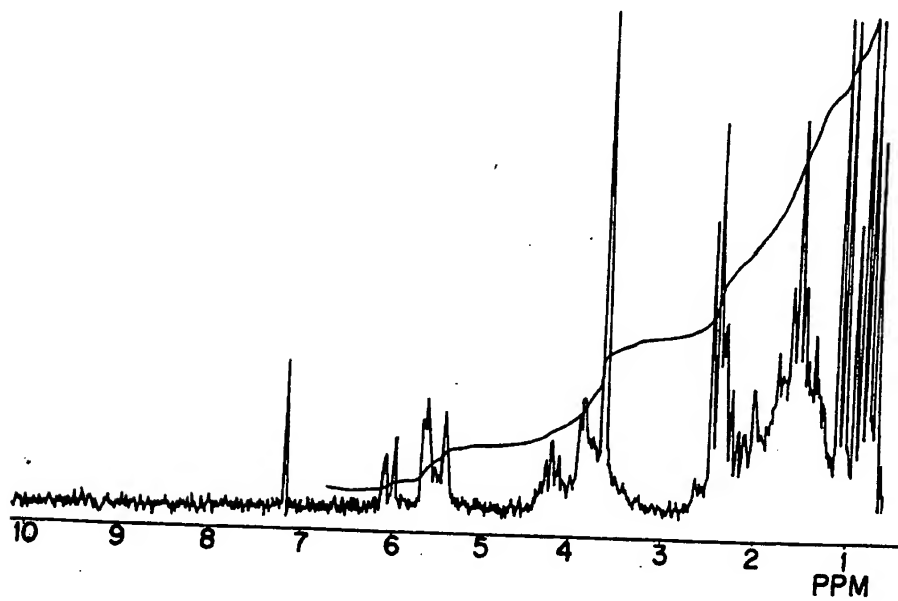


Fig 3

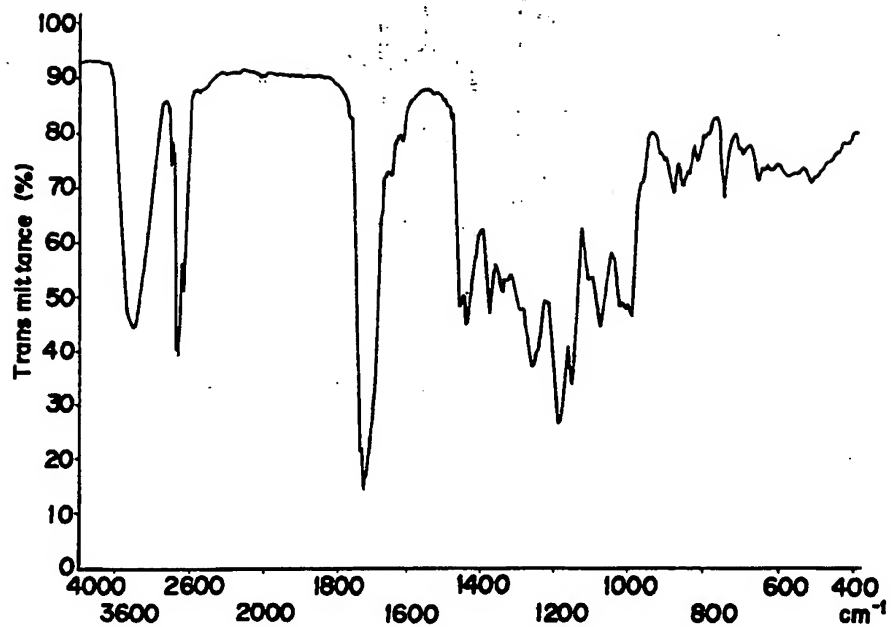


Fig 4

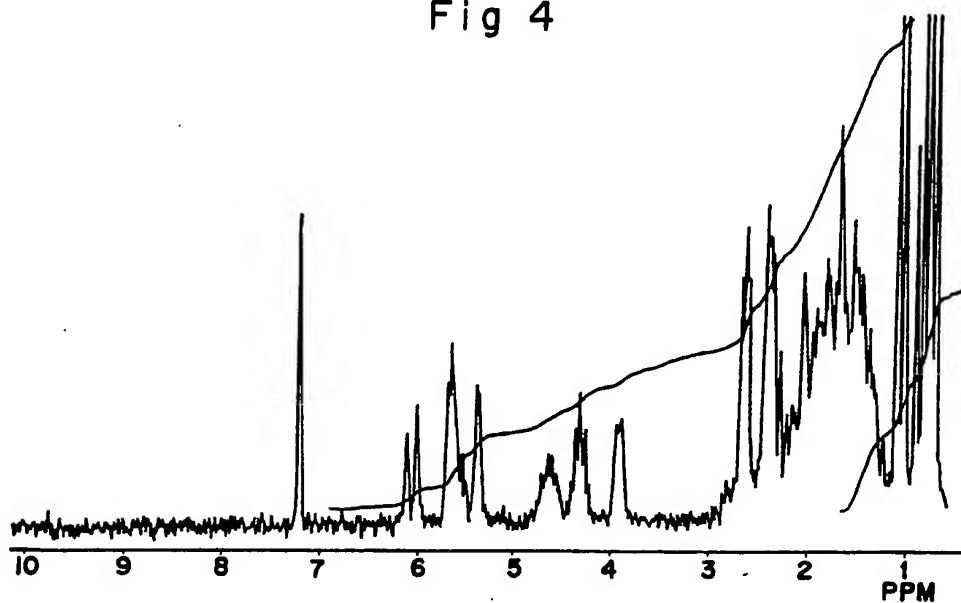
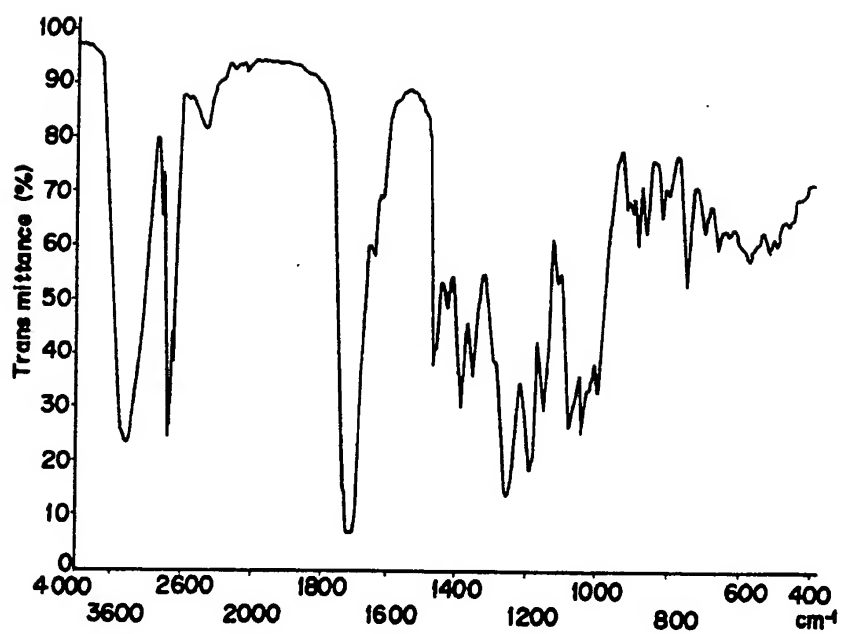


Fig 5

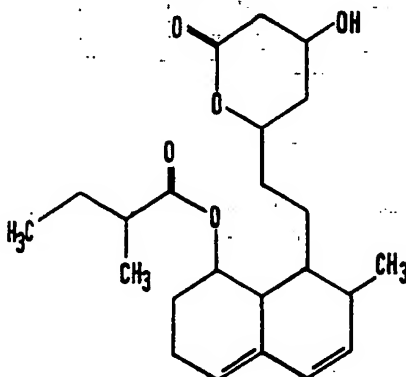


SPECIFICATION

ML—236B derivatives and their preparation

The present invention relates to a series of new derivatives of the known compound ML—236B, to processes for their preparation and to pharmaceutical compositions containing them.

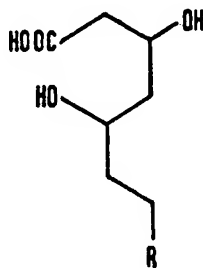
ML—236B, which has the following chemical structure:



is disclosed in U.S. Patent Specification No. 3,983,140. It has been isolated and purified from the metabolic products of microorganisms of the genus *Penicillium*, especially *Penicillium citrinum*, a species of blue mould. It has been shown to inhibit the biosynthesis of cholesterol by enzymes or cultured cells separated from experimental animals by competing with the rate-limiting enzyme active in the biosynthesis of cholesterol, namely 3-hydroxy-3-methylglutaryl-coenzyme A reductase and, as a result, significantly reduces serum cholesterol levels of animals [Journal of Antibiotics, 29, 1346 (1976)]. A number of compounds structurally related to ML—236B have also been discovered and found to possess the ability to inhibit the biosynthesis of cholesterol.

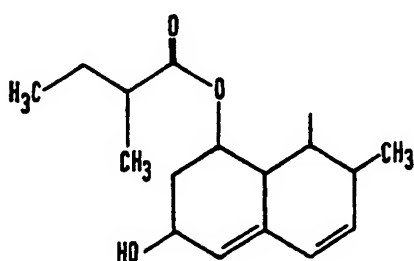
We have now discovered a series of new compounds, which may be prepared by the enzymatic hydroxylation of ML—236B or of derivatives thereof, and which possess an ability to inhibit the biosynthesis of cholesterol which is at least comparable with, and in some instances substantially exceeds, that of ML—236B itself.

The compounds of the present invention are those hydroxycarboxylic acids of formula (I):

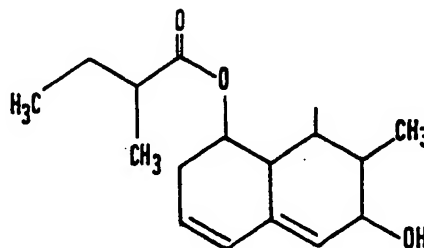


(I)

(in which R represents a group of formula



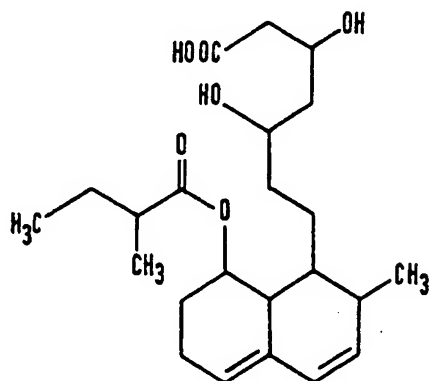
or



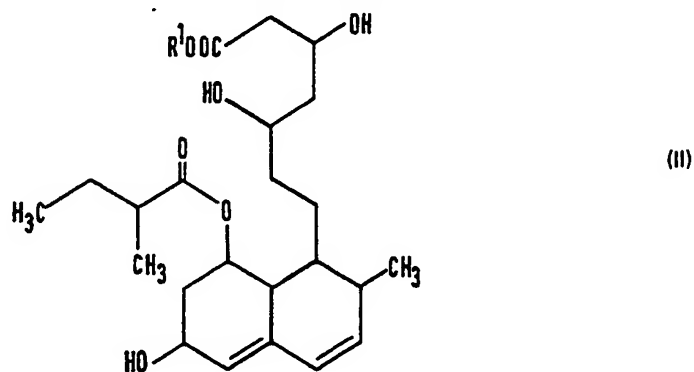
and ring-closed lactones, salts and esters thereof.

The invention also provides a process for preparing a compound of formula (I), or a ring-closed lactone, salt or ester thereof by the enzymatic hydroxylation of ML—236B, or ML—236B carboxylic acid, or a salt or ester thereof.

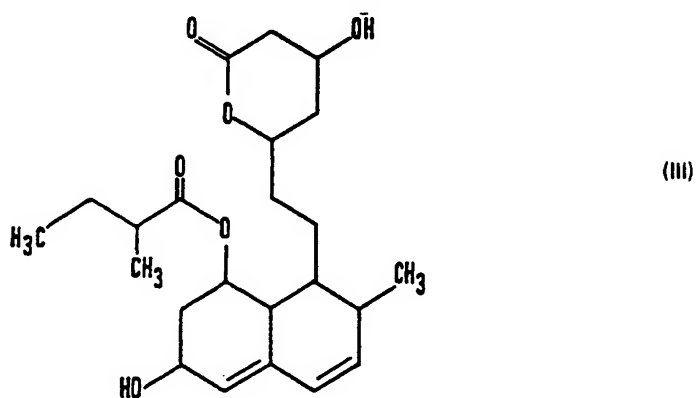
ML—236B carboxylic acid has the formula



One class of compounds of the present invention are those compounds of formula (II):

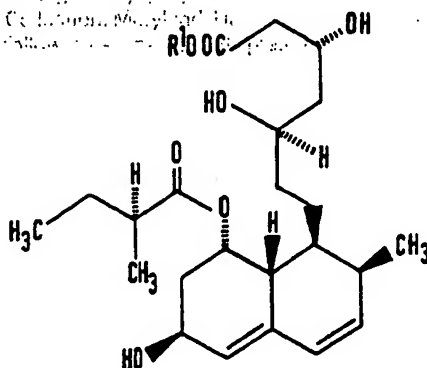


- 5 (in which R¹ represents a hydrogen atom or a C₁—C₈ alkyl group), pharmaceutically acceptable salts of the acid wherein R¹ represents a hydrogen atom, and the corresponding lactone of formula (III): 5



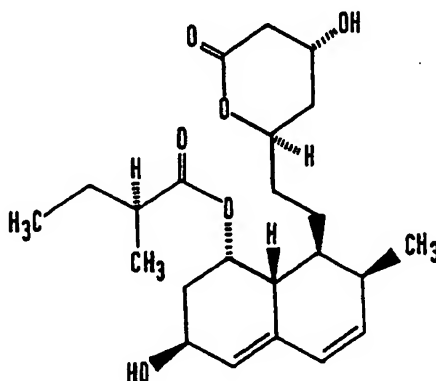
In view of the number of asymmetric carbon atoms in these compounds, a variety of geometric isomers are possible. Of these, the most important isomers are as follows:

Compounds of formula (IV):



(IV)

(in which R¹ is as defined above) and pharmaceutically acceptable salts of the acid wherein R¹ represents a hydrogen atom, and the corresponding lactone of formula (V):

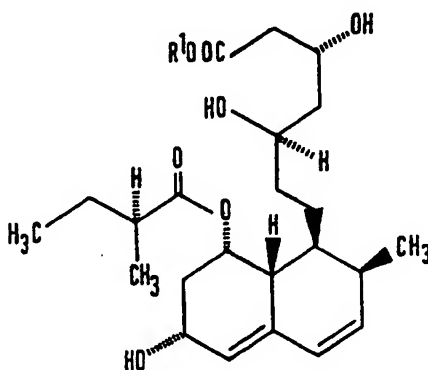


(V)

5

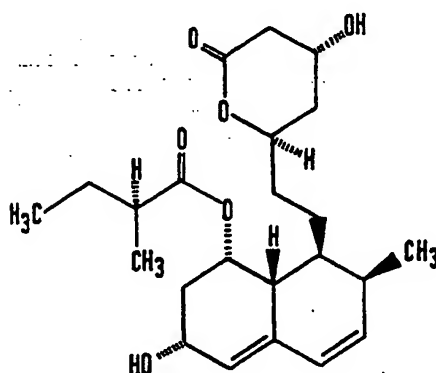
5

and compounds of formula (VI):



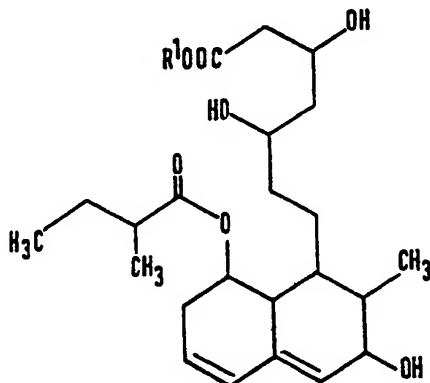
(VI)

(in which R¹ is as defined above), and pharmaceutically acceptable salts of the acid wherein R¹ represents a hydrogen atom, and the corresponding lactone of formula (VII):

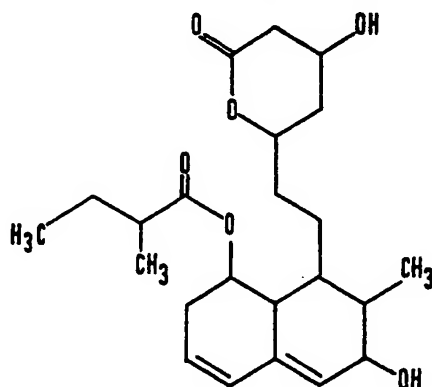


The hydroxy-carboxylic acid of formula (IV) in which R¹ represents a hydrogen atom is herein referred to as M—4 and derivatives of this acid, specifically the salts and esters, are named as derivatives of M—4, whilst the corresponding lactone of formula (V) is herein referred to as M—4 lactone. Similarly, the hydroxy-carboxylic acid of formula (VI) in which R¹ represents a hydrogen atom is referred to as M—4' and derivatives of this acid are referred to as derivatives of M—4', whilst the corresponding lactone of formula (VII) is referred to as M—4' lactone.

Another preferred class of compounds of the invention are those compounds of formula (VIII):

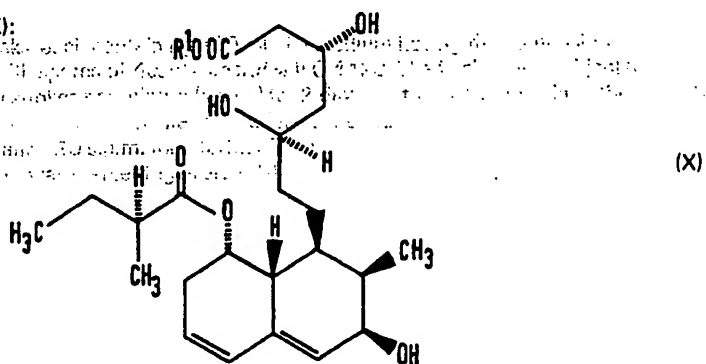


10 (in which R¹ is as defined above), and pharmaceutically acceptable salts of the acid in which R¹ represents a hydrogen atom, and the corresponding lactone of formula (IX):

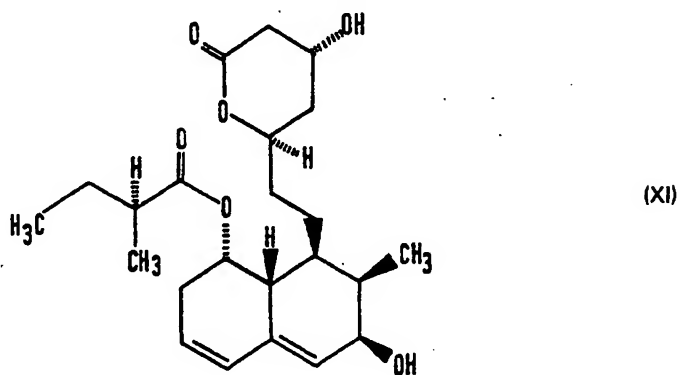


A variety of geometric isomers of these compounds are also possible, the most important being the following:

Compounds of formula (X):

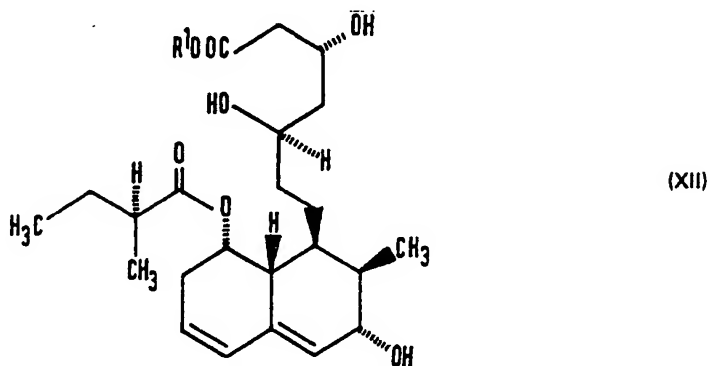


(in which R¹ is as defined above), and pharmaceutically acceptable salts of the acid in which R¹ represents a hydrogen atom and the corresponding lactone of formula (XI):

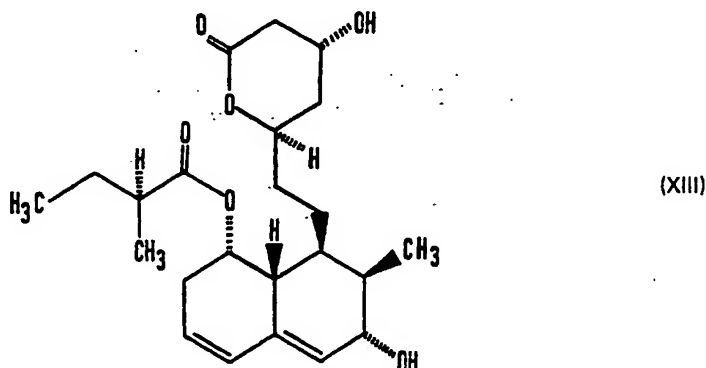


5 and compounds of formula (XII):

5



(in which R¹ is as defined above), and pharmaceutically acceptable salts of the acid in which R¹ represents a hydrogen atom and the corresponding lactone of formula (XIII):



The acid of formula (X) is herein referred to as IsoM—4 and its derivatives, such as salts and esters, are named as derivatives of IsoM—4, whilst the corresponding lactone of formula (XI) is herein referred to as IsoM—4 lactone. The acid of formula (XII) in which R¹ represents a hydrogen atom is herein referred to as IsoM—4', and its derivatives are named as derivatives of IsoM—4', whilst its corresponding lactone of formula (XIII) is herein referred to as IsoM—4' lactone.

Of the esters of the hydroxy-carboxylic acids of formula (I), the C₁—C₈ alkyl esters are preferred. These alkyl groups may be straight or branched-chain groups and include, for example the methyl, ethyl, propyl, isopropyl, butyl and isobutyl groups, of which the methyl group is particularly preferred.

The hydroxy-carboxylic acids will also form salts with a variety of cations, particularly metals and most preferably alkali metals, such as sodium or potassium. The sodium salts are most preferred.

Of the compounds of the invention, the most preferred compounds are M—4 lactone, M—4 sodium salt, M—4 methyl ester, IsoM—4' lactone, IsoM—4' sodium salt and IsoM—4' methyl ester, M—4 sodium salt being particularly preferred.

The compounds of the invention may be prepared by the enzymatic hydroxylation of ML—236B or of a derivative thereof, specifically ML—236B carboxylic acid or a salt or ester thereof.

This enzymatic hydroxylation may be effected as part of the mammalian metabolism of ML—236B or a derivative thereof, for example by administering ML—236B to a suitable animal, collecting a metabolic product, e.g. urine, and then separating the desired compound or compounds of the invention from this metabolic product. Alternatively, the liver or an enzyme-containing extract from the liver may be used instead of the living animal. However, processes employing the animal metabolism or animal products have a relatively low productivity and are difficult to carry out reproducibly. Accordingly, we prefer to employ microorganisms or enzyme-containing extracts from their microorganisms.

Accordingly, the process of the present invention is preferably effected using a microorganism capable of converting ML—236B or a derivative thereof to a compound of the present invention or using an enzyme-containing extract of such a microorganism. Particularly preferred microorganisms are those of the following genera: Mucor, Rhizopus, Zygorhynchus, Circinella, Actinomucor, Gongronella, Phycomyces, Martierella, Pycnoporus, Rhizoctonia, Absidia, Cunninghamhamella, Syncephalastrum and Streptomyces. In particular the following species are preferred:

Absidia coerulea
Cunninghamhamella echinulata
Syncephalastrum racemosum
Streptomyces roseochromogenus
Mucor hiemalis f. hiemalis
Mucor bacilliformis
Mucor circinelloides f. circinelloides
Mucor hiemalis f. corticolus
Mucor dimorphosporus
Mucor fragilis
Mucor genevensis
Mucor globosus
Mucor circinelloides f. griseo-cyanus
Mucor heterosporus
Mucor spinescens
Rhizopus chinensis
Rhizopus circinans
Rhizopus arrhizus
Zygorhynchus moelleri
Circinella muscae
Circinella rigida

- Circinella umbellata*
Actinomucor elegans
Phycomyces blakesleeanus
Martierella isabellina
5 *Gongronella butleri*
Pycnoporus coccineus
Rhizoctonia solani
Syncephalastrum nigricans
Absidia glauca var. *paradoxa*
10 Amongst strains of the above species, the following are particularly preferred: 10
Absidia coerulea IFO—4423
Cunninghamella echinulata IFO—4445
Cunninghamella echinulata IFO—4444
Cunninghamella echinulata ATCC—9244
15 *Syncephalastrum racemosum* IFO—4814 15
Syncephalastrum racemosum IFO—4828
Streptomyces roseochromogenus NRRL—1233
Streptomyces roseochromogenus IFO—3363
Streptomyces roseochromogenus IFO—3411
20 *Mucor hiemalis* f. *hiemalis* IFO—5834 20
Mucor hiemalis f. *hiemalis* IFO—5303
Mucor hiemalis f. *hiemalis* IFO—8567
Mucor hiemalis f. *hiemalis* IFO—8449
Mucor hiemalis f. *hiemalis* IFO—8448
25 *Mucor hiemalis* f. *hiemalis* IFO—8565 25
Mucor hiemalis f. *hiemalis* CBS—117.08
Mucor hiemalis f. *hiemalis* CBS—109.19
Mucor hiemalis f. *hiemalis* CBS—200.28
Mucor hiemalis f. *hiemalis* CBS—242.35
30 *Mucor hiemalis* f. *hiemalis* CBS—110.19 30
Mucor hiemalis f. *hiemalis* CBS—201.65
Mucor bacilliformis NRRL—2346
Mucor circinelloides f. *circinelloides* IFO—4554
Mucor circinelloides f. *circinelloides* IFO—5775
35 *Mucor hiemalis* f. *corticulus* NRRL—12473 35
Mucor dimorphosporus IFO—4556
Mucor fragilis CBS—236.35
Mucor genevensis IFO—4585
Mucor globosus NRRL 12474
40 *Mucor circinelloides* f. *griseo-cyanus* IFO—4563 40
Mucor heterosporus NRRL—3154
Mucor spinescens IAM—6071
Rhizopus chinensis IFO—4772
Rhizopus circinans ATCC—1225
45 *Rhizopus arrhizus* ATCC—11145 45
Zygorynchus moelleri IFO—4833
Circinella muscae IFO—4457
Circinella rigida NRRL—2341
Circinella umbellata NRRL—1713
50 *Circinella umbellata* IFO—4452 50
Circinella umbellata IFO—5842
Phycomyces blakesleeanus NRRL—12475
Martierella isabellina IFO—6739
Gongronella butleri IFO—8080
55 *Pycnoporus coccineus* NRRL—12476 55
Rhizoctonia solani NRRL—12477
Syncephalastrum nigricans NRRL—12478
Syncephalastrum nigricans NRRL—12479
Syncephalastrum nigricans NRRL—12480
60 *Absidia glauca* var. *paradoxa* IFO—4431 60
Actinomucor elegans ATCC—6476

The microorganisms listed above are available from International Culture Collections, as indicated by the codes appended to their accession numbers, which codes have the following meanings.

IFO = Institute for Fermentation, Osaka, Japan

65 NRRL = Agricultural Research Culture Collection, Illinois, USA 65

CBS = Centraal Bureau voor Schimmelcultures, Netherlands

IAM = Institute of Applied Microbiology, Tokyo, Japan

ATCC = American Type Culture Collection, Maryland, USA.

Of the species noted above, the following are particularly preferred:

5	<i>Absidia coerulea</i>	5
	<i>Cunninghamella echinulata</i>	
	<i>Syncephalastrum racemosum</i>	
	<i>Mucor hiemalis f. hiemalis</i>	
	<i>Mucor bacilliformis</i>	
10	<i>Mucor circinelloides f. circinelloides</i>	10
	<i>Mucor hiemalis f. corticolus</i>	
	<i>Mucor dimorphosporus</i>	
	<i>Mucor fragilis</i>	
	<i>Mucor genevensis</i>	
15	<i>Mucor globosus</i>	15
	<i>Mucor circinelloides f. griseo-cyanus</i>	
	<i>Mucor heterosporus</i>	
	<i>Mucor spinescens</i>	
	<i>Pycnoporus coccineus</i>	
20	<i>Rhizoctonia solani</i>	20
	<i>Syncephalastrum nigricans</i>	
	and the following are particularly preferred strains of the species:	
	<i>Absidia coerulea</i> IFO—4423	
	<i>Cunninghamella echinulata</i> IFO—4445	
25	<i>Cunninghamella echinulata</i> IFO—4444	25
	<i>Cunninghamella echinulata</i> ATCC—9244	
	<i>Syncephalastrum racemosum</i> IFO—4814	
	<i>Syncephalastrum racemosum</i> IFO—4828	
	<i>Mucor hiemalis f. hiemalis</i> IFO—5834	
30	<i>Mucor hiemalis f. hiemalis</i> IFO—5303	30
	<i>Mucor hiemalis f. hiemalis</i> IFO—8567	
	<i>Mucor hiemalis f. hiemalis</i> IFO—8449	
	<i>Mucor hiemalis f. hiemalis</i> IFO—8448	
	<i>Mucor hiemalis f. hiemalis</i> IFO—8565	
35	<i>Mucor hiemalis f. hiemalis</i> CBS—117.08	35
	<i>Mucor hiemalis f. hiemalis</i> CBS—109.19	
	<i>Mucor hiemalis f. hiemalis</i> CBS—200.28	
	<i>Mucor hiemalis f. hiemalis</i> CBS—242.35	
	<i>Mucor hiemalis f. hiemalis</i> CBS—110.19	
40	<i>Mucor hiemalis f. hiemalis</i> CBS—201.65	40
	<i>Mucor bacilliformis</i> NRRL—2346	
	<i>Mucor circinelloides f. circinelloides</i> IFO—4554	
	<i>Mucor circinelloides f. circinelloides</i> IFO—5775	
	<i>Mucor hiemalis f. corticolus</i> NRRL—12473	
45	<i>Mucor dimorphosporus</i> IFO—4556	45
	<i>Mucor fragilis</i> CBS—236.35	
	<i>Mucor genevensis</i> IFO—4585	
	<i>Mucor globosus</i> NRRL—12474	
	<i>Mucor circinelloides f. griseo-cyanus</i> IFO—4563	
50	<i>Mucor heterosporus</i> NRRL—3154	50
	<i>Mucor spinescens</i> IAM—6071	
	<i>Pycnoporus coccineus</i> NRRL—12476	
	<i>Rhizoctonia solani</i> NRRL—12477	
	<i>Syncephalastrum nigricans</i> NRRL—12478	
55	<i>Syncephalastrum nigricans</i> NRRL—12479	55
	<i>Syncephalastrum nigricans</i> NRRL—12480	
	For the preparation of compounds of formulae (IV) and (V) and their salts, the following species are preferred:	
	<i>Mucor hiemalis f. hiemalis</i>	
60	<i>Mucor circinelloides f. circinelloides</i>	60
	<i>Mucor fragilis</i>	
	<i>Mucor genevensis</i>	
	<i>Mucor circinelloides f. griseo-cyanus</i>	
	<i>Pycnoporus coccineus</i>	
65	<i>Rhizoctonia solani</i> .	65

For the preparation of compounds of formula (VI) and (VIII) and their salts, the species *Syncephalastrum nigricans* and *Syncephalastrum racemosum* are preferred.

For the preparation of compounds of formula (VIII) and (IX) and their salts, the species *Absidia coerulea* and *Cunninghamella echinulata* are preferred.

Of all of the species listed above, *Mucor hiemalis f. hiemalis* is particularly preferred since it is able to convert ML—236B and its derivatives to the desired compounds of formula (I) at a conversion of 90% or even higher.

Conversion of ML—236B or derivatives thereof to compounds of formula (I) may be achieved by contacting the complete cellular microorganism or, in some cases, a cell-free extract from the microorganism with ML—236B or a derivative thereof. The form of the compound produced will depend upon the culture conditions and the form of microorganism employed. Thus, for example, if the complete cellular microorganism is cultivated in the presence of ML—236B or a derivative thereof, the product will be the carboxylic acid, the lactone or alkali metal salt, depending upon the culture conditions, particularly the pH. On the other hand, if the ML—236B or derivative thereof is simply contacted with a resting cellular system or with a cell-free extract, the compound of the invention is obtained in the form of an alkali metal salt.

The progress of the conversion reaction may be determined by assaying samples of the reaction mixture during the course of the reaction to determine the degree of conversion. For example, the presence of M—4 lactone may be assayed by liquid chromatography employing as a carrier Micro Bondapak C₁₈ (manufactured by Waters Co. USA) and as the solvent 62% v/v aqueous methanol at the rate of 1 ml/minute. When detected using its ultraviolet absorption at 237 nm, M—4 gives a peak at a retention time of 10 minutes, and this may be used for the assay. Similar techniques are available for assaying the other compounds of the invention.

Where the microorganisms are to be cultivated in the presence of ML—236B or a derivative thereof to produce the compounds of the invention, the culture conditions and culture media employed will be chosen having regard to the particular microorganism to be cultivated. Since the species of microorganism proposed for use in the process of the present invention are well known, culture conditions and culture media for use with these microorganisms are also well known.

The compounds of the invention may be separated from the reaction mixture by conventional means, for example by filtering off microbial cells (if necessary) and then subjecting the remaining mixture to any combination of thin layer chromatography, column chromatography or high performance liquid chromatography. The various compounds of the invention, where two or more are prepared together, may be separated from each other in the course of one or more of these chromatographic purification steps.

In addition to the compounds of the invention, there may, in some cases, also be prepared a compound which we have designated M—3 and which is known under the name 3',5'-dihydroxy-(dihydro-ML—236B) in a copending application entitled "Hydronephthalene Derivatives, their Preparation and Use". This may also be separated in the same way.

We have found that the compounds of the invention give a 50% inhibition of cholesterol biosynthesis at concentrations comparable with, or, in some cases, significantly less than, the concentrations required by ML—236B and certain other similar known compounds. The inhibitory activities of certain of the compounds of the invention, in terms of the concentration in µg/ml required to inhibit cholesterol biosynthesis by 50% [measured by the method described in the Journal of Biological Chemistry, 234, 2835 (1959)] are as follows:

45	M—4 methyl ester	0.001	45
	M—4 sodium salt	0.0008	
	M—4 lactone	0.016	
	IsoM—4' methyl ester	0.007	
	IsoM—4' lactone	0.013	
50	M—4'	0.019	50
	M—4' sodium salt	0.00049	
	ML—236B	0.01	

The invention is further illustrated by the following Examples.

EXAMPLE 1

Preparation of M—4 lactone

Twenty 500 ml Sakaguchi flasks, each containing 100 ml of a medium having the composition described below, were inoculated with spores of *Absidia coerules* IFO 4423. The flasks were subjected to shaking culture at 26°C and 120 strokes per minute (s.p.m.) for 2 days. At the end of this time, the sodium salt of ML—236B was added to each of the flasks to a final concentration of 0.05% w/v. Cultivation was continued at 26°C and 120 s.p.m. for a further 5 days.

The composition of the medium was (percentages are w/v):

	Glucose	2.0%	
10	K ₂ HPO ₄	0.15%	10
	MgSO ₄ ·7H ₂ O	0.15%	
	NH ₄ NO ₃	0.1%	
	Peptone	0.1%	
	Corn steep liquor	0.2%	
15	Yeast extract	0.1%	15
	ZnSO ₄ ·7H ₂ O	0.001%	
	Tap water	the balance (adjusted to pH 7.0).	

After completion of the cultivation, the reaction liquor was filtered, and the filtrate was adjusted with trifluoroacetic acid to pH 3. The resulting mixture was extracted with three 1 litre portions of ethyl acetate, to give extracts containing M—4. This compound shows an R_f value of 0.45 on thin layer chromatography (TLC) (Plate: Merck silica gel Art 5715; solvent: a 50:50:3 by volume mixture of benzene, acetone and acetic acid). The combined extracts were washed with saturated aqueous sodium chloride, and then a catalytic amount of trifluoroacetic acid was added for lactonization. The resulting mixture was then washed with a 1% w/v aqueous solution of sodium bicarbonate, dried over anhydrous sodium sulphate and evaporated under reduced pressure to dryness. The residue was subjected to preparative liquid chromatography, System 500 using a Prep PAK—500/C₁₈ cartridge manufactured by Waters Associates (Prep PAK is a Trade Mark). Purification with a 55% v/v aqueous methanol system yielded 50.1 mg of M—4 lactone.

M—4 lactone has the following physical properties.

1) Nuclear Magnetic Resonance Spectrum:

The NMR spectrum measured at 60 MHz in deuterochloroform using tetramethylsilane as the internal standard is shown in Figure 1 of the accompanying drawings.

2) Ultraviolet absorption spectrum (methanol solution) λ_{max} nm: 230; 236.7; 244.6.

3) Infrared absorption spectrum (liquid film) ν cm⁻¹: 3400, 2950, 1725.

4) Thin layer chromatography:

TLC plate: Merck silica gel Art 5715;

Solvent: benzene, acetone, acetic acid (50:50:3 by volume);

R_f value: 0.62.

EXAMPLE 2

48 mg of M—4 lactone were prepared following the same procedures as in Example 1, but using *Cunninghamella echinulata* IFO 4445.

EXAMPLE 3

30 mg of M—4 lactone were prepared following the same procedures as in Example 1, but using *Streptomyces roseochromogenus* NRRL 1233.

EXAMPLE 4

5 mg of M—4 lactone were prepared following the same procedures as in Example 1, but using *Syncephalastrum racemosum* IFO 4814.

EXAMPLE 5

6 mg of M—4 lactone were prepared following the same procedures as in Example 1, but using *Syncephalastrum racemosum* IFO 4828.

EXAMPLE 6**Preparation of IsoM—4' methyl ester**

Twenty 500 ml Sakaguchi flasks, each containing 100 ml of a medium having the composition described below, were inoculated with spores of *Absidia coerulea* IFO 4423. The flasks were subjected to shaking culture at 120 s.p.m. and 26°C for 2 days. At the end of this time, the sodium salt of ML—236B was added to each of the flasks to a final concentration of 0.05% w/v. Cultivation was continued at 120 s.p.m. and 26°C for a further 5 days.

The composition of the medium was (percentages are w/v):

	Glucose	2.0%	
10	K ₂ HPO ₄	0.15%	10
	MgSO ₄ ·7H ₂ O	0.15%	
	NH ₄ NO ₃	0.1%	
	Peptone	0.1%	
	Corn steep liquor	0.2%	
15	Yeast extract	0.1%	15
	ZnSO ₄ ·7H ₂ O	0.001%	
	Tap water	the balance (adjusted to pH 7.0).	

After completion of the cultivation, the reaction liquor was filtered, and the filtrate was adjusted with trifluoroacetic acid to pH 3. The resulting mixture was extracted with three 1 litre portions of ethyl acetate to give extracts containing IsoM—4'. This compound has an R_f value of 0.45 on thin layer chromatography (plate: Merck silica gel Art 5715; solvent: a 50:50:3 by volume mixture of benzene, acetone and acetic acid). The extract was washed with a saturated aqueous solution of sodium chloride, and then an ethereal solution of diazomethane was added. The mixture was allowed to stand for 30 minutes and then evaporated under reduced pressure to dryness. The residue was placed on a Lobar column (Merck Si 60, Size A) and purified using as the solvent system a 1:1 by volume mixture of benzene and ethyl acetate. There were obtained 200 mg of an IsoM—4' methyl ester fraction. This fraction was further purified on a Lobar column (Merck RP—8, Size A) using 35% v/v aqueous acetonitrile as the eluent to give 78 mg of pure IsoM—4' methyl ester, having the following characteristics:

1) Nuclear Magnetic Resonance Spectrum:

The NMR spectrum measured at 100 MHz in deuteriochloroform using tetramethylsilane as the internal standard is shown in Figure 2 of the accompanying drawings.

2) Mass spectrum:

Measurement was made [after silylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide] using a mass spectrometer, type D—300 manufactured by Nippon Electronics.

M/e: 654 (M⁺), 552, 462, 372, 272, 233, 231.

3) Ultraviolet absorption spectrum (methanol solution) λ_{\max} nm: 229, 234.8; 244.5.

4) Infrared absorption spectrum (liquid film): As shown in Figure 3 of the accompanying drawings.

5) Thin layer chromatography:

TLC plate: Merck silica gel Art 5715;

Solvent: benzene, acetone (1:1 by volume);

R_f value: 0.88.

By operating as described above but replacing the diazomethane by another appropriate diazoalkane, it is possible to produce other esters of IsoM—4'.

EXAMPLE 7**Preparation of IsoM—4' lactone**

The procedure described in Example 6 was repeated up to and including extraction with ethyl acetate to give extracts containing IsoM—4'. The combined extracts were washed with a saturated aqueous solution of sodium chloride and then evaporated to dryness to give the lactone product. The resulting residue was placed on a Lobar column (Merck Si 60, Size A) and purified using as the solvent system a 1:1 by volume mixture of benzene and ethyl acetate, to afford 198 mg of IsoM—4' lactone.

This product was further purified by means of a Lobar column (Merck RP—8, Size A) eluted with 35% v/v aqueous acetonitrile, to give 82 mg of pure IsoM—4 lactone, having the following characteristics:

1) Nuclear Magnetic Resonance Spectrum:

The NMR spectrum measured at 100 MHz in deuteriochloroform using tetramethylsilane as the internal standard is shown in Figure 4 of the accompanying drawings.

2) Ultraviolet absorption spectrum (methanol solution) λ_{\max} nm: 229, 234.8; 244.5.

3) Infrared absorption spectrum (liquid film): As shown in Figure 5 of the accompanying drawings.

EXAMPLE 8

63 mg of IsoM—4' lactone were prepared, following the same procedures as in Example 7, but using *Cunninghamella echinulata* IFO 4445.

EXAMPLE 9

24 mg of IsoM—4' lactone were prepared, following the same procedures as in Example 7, but using *Syncephalastrum racemosum* IFO 4814.

EXAMPLE 10

35 mg of IsoM—4' lactone were prepared, following the same procedures as in Example 7, but using *Syncephalastrum racemosum* IFO 4828.

EXAMPLE 11

12 mg of IsoM—4' lactone were produced according to the process described in Example 7, but using *Streptomyces roseochromogenus* NRRL 1233.

EXAMPLE 12

Preparation of IsoM—4' sodium salt

In a small amount of acetone were dissolved 10 mg of IsoM—4' lactone. To the solution was added an equivalent amount of sodium hydroxide and the mixture was allowed to stand for 1 hour. The pH of the resulting mixture was adjusted with 0.1N hydrochloric acid to a value of 8.0. The acetone was then distilled off, and the residue was placed on an XAD—20 column (about 20 ml). The column was washed with distilled water and then eluted with 50 ml of 50% v/v aqueous acetone. The acetone was again distilled off, and the residue was freeze-dried to afford 6 mg of IsoM—4' sodium salt, having the following characteristics:

1) Ultraviolet absorption spectrum (methanol solution) λ_{\max} nm: 229 (shoulder); 235, 245 (shoulder).

2) Infrared absorption spectrum (KBr) ν cm⁻¹: 3400, 2850, 1710, 1580.

3) Thin layer chromatography:

TLC plate: Merck silica gel Art 5715;

Solvent: benzene, acetone, acetic acid (50:50:3 by volume);

Rf value: 0.45.

EXAMPLE 13

Preparation of M—4 methyl ester

Twenty 500 ml Sakaguchi flasks, each containing 100 ml of a medium of the same composition as shown in Example 1, were inoculated with spores of *Absidia coerulea* IFO 4423. The flasks were subjected to shaking culture at 26°C and 120 s.p.m. for 2 days. The sodium salt of ML—236B was then added to each of the flasks to a final concentration of 0.05 w/v. Cultivation was continued at 26°C and 120 s.p.m. for a further 5 days.

After completion of the cultivation, the reaction liquor was filtered, and the filtrate was adjusted with trifluoroacetic acid to pH 3. The resulting mixture was extracted with three 1 litre portions of ethyl acetate, to give extracts containing M—3, M—4 and IsoM—4'. Both M—4 and IsoM—4' show an Rf value of 0.45 on thin layer chromatography (Plate: Merck silica gel Art 5715; solvent: a 50:50:3 by volume mixture of benzene, acetone and acetic acid). The combined extracts were washed with saturated aqueous sodium chloride, and then an ethereal solution of diazomethane was added. The mixture was allowed to stand for 30 minutes and then evaporated under reduced pressure to dryness. When the residue was placed on a Lobar column (Merck Si 60, Size A), and purification was effected using a 1:1 by volume mixture of benzene and ethyl acetate, a fraction containing IsoM—4' methyl ester and a fraction containing M—4 methyl ester were separated. There were obtained 185.3 mg of the latter active fraction, from which 20 mg of pure M—4 methyl ester were obtained as a colourless oil by using a Lobar column (Merck RP—8, Size A) and eluting with 35% v/v aqueous acetonitrile.

M—4 methyl ester has the following characteristics:

1) Nuclear Magnetic Resonance Spectrum:

Measurement was made at 200 MHz in deuteriochloroform using tetramethylsilane as the internal standard.

- δ ppm:
 0.88 (3H, triplet, $J = 7.3$ Hz);
 0.89 (3H, doublet, $J = 6.5$ Hz);
 1.12 (3H, doublet, $J = 6.8$ Hz);
 1.1—1.7 (10H, multiplet);
 2.34 (1H, sextuplet, $J = 7$ Hz);
 2.3—2.5 (2H, multiplet);
 2.49 (2H, doublet, $J = 6.4$ Hz);
 2.58 (1H, multiplet);
 3.72 (3H, singlet);
 3.78 (1H, multiplet);
 4.25 (1H, quintet, $J = 7$ Hz);
 4.4 (1H, multiplet);
 5.42 (1H, multiplet);
 5.56 (1H, multiplet);
 5.90 (1H, doubled doublet, $J = 9.8$ and 5.6 Hz);
 5.99 (1H, doublet, $J = 9.8$ Hz).
 2) Mass spectrum:
 Measurement was made [after silylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide] using a
 mass spectrometer, type D—300 manufactured by Nippon Electronics.
- M/e: 654(M^+), 552, 462, 372, 290, 272, 233, 231.
- 3) Ultraviolet absorption spectrum (ethanol solution) λ_{\max} nm: 230.1; 237.3; 246.4.
 4) Infrared absorption spectrum (liquid film) ν cm^{-1} : 3400, 2950, 1730.
 5) Thin layer chromatography:
 TLC plate: Merck silica gel Art 5715;
 Solvent: benzene and acetone (1:1 by volume);
 Rf value: 0.88.
 By operating as described above but replacing the diazomethane by another appropriate
 diazoalkane, it is possible to produce other esters of M—4.
- 30 EXAMPLE 14**
- Preparation of Sodium Salts of M—4 and IsoM—4'
- The procedure described in Example 1 was repeated (except that the culture medium contained Na_2HPO_4 instead of K_2HPO_4) up to and including filtration of the reaction liquor. The filtrate was then adsorbed on an HP—20 column (manufactured by Mitsubishi Chemical Industries). After washing the column with water, fractions containing M—4 sodium salt, IsoM—4' sodium salt and M—3 sodium salt were eluted with 50% v/v aqueous acetone. The active fractions were freeze-dried, giving 830 mg of a freeze-dried product, which was purified by repeatedly subjecting it to high-performance liquid chromatography (column: Micro Bondapak C_{18} , 40% v/v aqueous methanol 1 ml/min.) to give 32 mg of M—4 sodium salt and 280 mg of IsoM—4' sodium salt.
- The properties of the IsoM—4' sodium salt were identical to those of the product of Example 12 and the properties of the M—4 sodium salt are as follows:
- 1) Nuclear Magnetic Resonance Spectrum:
 Measurement was made at 200 MHz in deuteromethanol using tetramethylsilane as the internal standard.
- δ ppm:
 0.91 (3H, triplet, $J = 7.5$ Hz);
 0.92 (3H, doublet, $J = 7$ Hz);
 1.12 (3H, doublet, $J = 7$ Hz);
 1.1—1.8 (10H, multiplet);
 2.25 (1H, doubled doublet, $J = 15$ and 7.6 Hz);
 2.34 (1H, doubled doublet, $J = 15$ and 5.5 Hz);
 2.2—2.4 (3H, multiplet);
 2.48 (1H, multiplet);
 3.68 (1H, multiplet);
 4.07 (1H, multiplet);
 4.28 (1H, multiplet);
 5.36 (1H, multiplet);
 5.48 (1H, doubled doublet, $J = 3$ and 2 Hz);
 5.88 (1H, doubled doublet, $J = 9.6$ and 5.3 Hz);
 5.98 (1H, doublet, $J = 9.8$ Hz).
 2) Ultraviolet absorption spectrum (methanol solution) λ_{\max} nm: 230.0; 237.2; 245.0.
 3) Infrared absorption spectrum (KBr) ν cm^{-1} : 3400, 2900, 1725, 1580.

4) Thin layer chromatography:
 TLC plate: Merck silica gel Art 5715;
 Solvent: benzene, acetone and acetic acid (50:50:3 by volume);
 Rf value: 0.45.

5 EXAMPLE 15

18 mg of M—4 methyl ester were prepared, following the same procedures as in Example 13, but using *Cunninghamella echinulata* IFO 4445.

EXAMPLE 16

33 mg of M—4 methyl ester were prepared, following the same procedures as in Example 13, but using *Streptomyces roseochromogenus* NRRL 1233.

EXAMPLE 17

12 mg of M—4 methyl ester were prepared, following the same procedures as in Example 13, but using *Syncephalastrum racemosum* IFO 4814.

EXAMPLE 18

16 mg of M—4 methyl ester were prepared, following the same procedures as in Example 13, but using *Syncephalastrum racemosum* IFO 4828.

EXAMPLE 19

Preparation of M—4 methyl ester

Five beagles (male, average weight 10 kg) were administered with ML—236B at a dose of 200 mg/kg/day and their urine was collected for 3 days. 3 litres of collected urine were passed through a 500 ml XAD—2 column, eluted with 500 ml of 50% v/v aqueous acetone, and, after distilling off the acetone under reduced pressure, the residual liquid was adjusted to pH 3 by the addition of trifluoroacetic acid. The mixture was then extracted three times, each time with 1 litre of ethyl acetate, to give M—4. This compound shows an Rf value of 0.45 on thin layer chromatography (TLC plate: Silica Gel Art 5715 manufactured by Merck & Co., Inc.; solvent: a 50:50:3 by volume mixture of benzene, acetone and acetic acid). The extract was washed with a saturated aqueous solution of sodium chloride, and, after adding an ethereal solution of diazomethane, left standing for 30 minutes. It was then evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml of a 55% v/v aqueous methanol solution, and passed through a column chromatograph (Product of Merck & Co., Inc.; RP—8, Size B). After passing 200 ml of a 55% v/v aqueous methanol solution, it was eluted with a 60% v/v aqueous methanol solution. The first 240 ml of the eluate were discarded, and the next 120 ml were collected. This fraction was evaporated to dryness and the residue was dissolved in 2.5 ml of a 65% v/v aqueous methanol solution and purified by high-performance liquid chromatography (JASCO—Trirotar, column: μ — Bondapak C₁₈). The portion which showed the fourth peak was separated and the solvent was distilled off to give M—4 methyl ester as a colourless oil having the properties shown in Example 13. 4 mg of product were obtained.

EXAMPLE 20

Preparation of M—4

Homogenized rabbit liver was used in this Example to obtain M—4 from ML—236B.

40 (a) Enzymatic solution

Three volumes of a 1.15% w/v potassium chloride — 10 mM phosphate (pH 7.4) buffer solution were added to one volume of rabbit liver and the mixture was homogenized. The homogenized mixture was then centrifuged for 20 minutes at 9,000 G and the supernatant fraction was taken as an enzymatic solution.

45 (b) Cofactor solution

β -Nicotinamide adenine dinucleotide phosphate (reduced form NADPH)	3 mg	
MgCl ₂ solution (508 mg/10 ml)	0.1 ml	
1.15% w/v KCl solution	0.3 ml	
0.2 M phosphate buffer solution (pH 7.4)	0.6 ml	

The above substances were mixed to a total volume of 1 ml to make the cofactor solution.

(c) Reaction solution

80 μ l of the above enzymatic solution, 20 μ l of the above cofactor solution and 2 μ l of a methanol solution of ML—236B were mixed to make a final concentration of ML—236B of 1 mM. The resulting solution was shaken for 30 minutes at 37°C. M—4 was formed in the reaction mixture and identified by TLC (the same conditions as in Example 19).

EXAMPLE 21

Preparation of M—4 sodium salt

2 mg of M—4 methyl ester were dissolved in 1 ml of a 0.1 N aqueous solution of sodium chloride and subjected to hydrolyzation at 30°C for 1 hour. The reaction mixture was washed with 1 ml of chloroform and the resulting aqueous phase was adjusted to pH 8 with 0.1 N hydrochloric acid and passed through a XAD—2 column (about 5 ml). The column was washed with 20 ml of distilled water and the desired product was eluted with 15 ml of 50% v/v aqueous acetone. The acetone was distilled off from the eluate. The residue was confirmed by high-performance liquid chromatography to give a single peak (retention time was 13 minutes, eluted with 40% v/v aqueous methanol at 1 ml/minute). The residue was then lyophilized to give 0.8 mg of M—4 Na salt having the same properties as the product of Example 14.

EXAMPLE 22

Preparation of M—4 methyl ester

Each of twenty 500 ml. Erlenmeyer flasks containing 100 ml. of a medium having the composition listed below was inoculated with spores of *Mucor hiemalis f. hiemalis* IFO—5834. The inoculum was subjected to shaking culture at 26°C. and 220 rpm. After 4 days, ML—236B was added to a final concentration of 0.05% w/v, and cultivation was conducted at 26°C. and 220 rpm for additional 6 days. The composition of the medium was (percentages are w/v):

Glucose	1.0%	
Peptone	0.2%	
Meat extract	0.1%	25
Yeast extract	0.1%	
Corn steep liquor	0.3%	
Tap water	balance (pH unadjusted).	

After completion of the cultivation, the filtrate was adjusted to a pH of 3 with trifluoroacetic acid. The mixture was then extracted three times, each time with 100 ml. of ethyl acetate. There was obtained a fraction containing M—4. M—4 has an R_f value of 0.45 on thin layer chromatography (Plate: Merck Silica gel Art 5715; Solvent: a 50:50:3 by volume mixture of benzene, acetone and acetic acid). The conversion ratio was 90%. This extract was washed with a saturated aqueous solution of sodium chloride, after which there was added an ethereal solution of diazomethane. The resulting mixture was allowed to stand for 30 minutes and then concentrated under reduced pressure to dryness. The residue was placed on a Lobar column (Merck Si 60, size A) and purified with a 1:1 by volume mixture of benzene and ethyl acetate. There were obtained about 600 mg. of M—4 methyl ester, having the same properties as the product of Example 13.

EXAMPLE 23

Preparation of M—4 lactone

The procedure described in Example 22 was repeated up to and including washing of the three ethyl acetate extracts with a saturated aqueous solution of sodium chloride. The resulting solution was then evaporated to dryness to give a lactone product. The product was recrystallized from ethyl acetate to give about 560 mg. (56%) of M—4 lactone, having the same properties as the product of Example 1.

EXAMPLE 24

Preparation of M—4 sodium salt

The procedure described in Example 22 was repeated to give 1.9 litres of the filtrate from the conversion reaction. This was extracted three times, each time with 1 litre of ethyl acetate to give fractions containing M—4. By immediately transferring these into a 5% w/v aqueous solution of sodium bicarbonate, there was obtained a fraction containing M—4 sodium salt. Then the M—4 sodium fraction was adjusted with 2N hydrochloric acid to a pH of 7.0 and adsorbed on an HP—20 column (manufactured by Mitsubishi Chemical Industries). Washing with water and elution with 50% v/v aqueous acetone gave a fraction containing M—4 sodium salt, from which there were obtained 570

mg. (52%) of a freeze-dried product, having the properties described in Example 14.

EXAMPLE 25

Preparation of M—4

The procedure described in Example 22 was repeated, except that the following microorganisms were employed and the conversion to M—4 was as shown by the associated codes:

	Microorganism:	Conversion to M—4	
	<i>Mucor hiemalis f. hiemalis</i> IFO—5303	+4	
	<i>Mucor hiemalis f. hiemalis</i> IFO—8567	+4	
	<i>Mucor hiemalis f. hiemalis</i> IFO—8449	+4	
10	<i>Mucor hiemalis f. hiemalis</i> IFO—8448	+4	10
	<i>Mucor hiemalis f. hiemalis</i> IFO—8565	+4	
	<i>Mucor hiemalis f. hiemalis</i> CBS—117.08	+4	
	<i>Mucor hiemalis f. hiemalis</i> CBS—109.19	+4	
	<i>Mucor hiemalis f. hiemalis</i> CBS—200.28	+4	
15	<i>Mucor hiemalis f. hiemalis</i> CBS—242.35	+4	15
	<i>Mucor hiemalis f. hiemalis</i> CBS—110.19	+4	
	<i>Mucor hiemalis f. hiemalis</i> CBS—201.65	+4	
	<i>Mucor bacilliformis</i> NRRL—2346	trace	
	<i>Mucor circinelloides f. circinelloides</i> IFO—4554	+1	
20	<i>Mucor circinelloides f. circinelloides</i> IFO—5775	+1	20
	<i>Mucor hiemalis f. corticolus</i> NRRL—12473	trace	
	<i>Mucor dimorphosporus</i> IFO—4556	trace	
	<i>Mucor fragilis</i> CBS—236.35	+1	
	<i>Mucor genevensis</i> IFO—4585	+1	
25	<i>Mucor globosus</i> NRRL—12474	trace	25
	<i>Mucor circinelloides f. griseo-cyanus</i> IFO—4563	+1	
	<i>Mucor heterosporus</i> NRRL—3154	trace	
	<i>Mucor spinescens</i> IAM—6071	trace	
	<i>Mucor chinensis</i> IFO—4772	trace	
30	<i>Rhizopus circinans</i> ATCC—1225	+1	30
	<i>Rhizopus arrhizus</i> ATCC—11145	+1	
	<i>Zygorynchus moelleri</i> IFO—4833	+1	
	<i>Circinella muscae</i> IFO—4457	+	
	<i>Circinella rigida</i> NRRL—2341	trace	

	Microorganism:	Conversion to M—4	
	<i>Circinella umbellata</i> NRRL—1713	+1	
	<i>Circinella umbellata</i> IFO—4452	+1	
	<i>Circinella umbellata</i> IFO—5842	+1	
5	<i>Actinomucor elegans</i> ATCC—6476	+1	5
	<i>Phycomyces blakesleeana</i> NRRL—12475	trace	
	<i>Martierella isabellina</i> IFO—6739	trace	
	<i>Gongronella butleri</i> IFO—8080	+1	
	<i>Pycnoporus coccineus</i> NRRL—12476	+3	
10	<i>Rhizoctonia solani</i> NRRL—12477	+2	10

The codes representing the conversions to M—4 have the following meanings:

	trace = 0.5% or less	
	+1 = 0.5—5%	
	+2 = 5.0—10.0%	
15	+3 = 10.0—30.0%	15
	+4 = 70.0—90.0%	

EXAMPLE 26

Preparation of IsoM—4' lactone

- Each of twenty 500 ml. Sakaguchi flasks containing 100 ml. of a medium of the composition described in Example 22 was inoculated with spores of *Circinella muscae* IFO—4457. The inoculum was subjected to shaking culture at 26°C. and 220 rpm. After 4 days, ML—236B was added to a final concentration of 0.05% w/v, and cultivation was conducted at 26°C. and 120 rpm for a further 6 days.

- After completion of the cultivation, the conversion reaction mixture was filtered, and the filtrate was adjusted with trifluoroacetic acid to a pH of 3.0. The mixture was then extracted three times, each time with 1 litre of ethyl acetate, giving a fraction containing IsoM—4'. This extract was washed with a saturated aqueous solution of sodium chloride and then evaporated to dryness. There was obtained a lactone product. The residue was placed on a Lobar column (Merck Si 60, size A) and purified with an ethyl acetate system, giving 12 mg. of IsoM—4' lactone, having the properties described in Example 7.

30 EXAMPLE 27

Preparation of M—4' lactone

- Each of twenty 500 ml. Erlenmeyer flasks containing 100 ml. of a medium having the composition shown below was inoculated with spores of *Syncephalastrum nigricans* NRRL—12478. The inoculum was subjected to shaking culture at 26°C. and 20 rpm. After 3 days, ML—236B was added to a final concentration of 0.05% w/v and cultivation was carried out at 26°C. and 220 rpm.

The composition of the medium was as follows (percentages are w/v):

	Glucose	1%	
	Peptone	0.2%	
	Meat extract	0.1%	
40	Yeast extract	0.1%	40
	Corn steep liquor	0.3%	
	(pH unadjusted).		

- After completion of the cultivation, the conversion reaction mixture was filtered, and the filtrate was adjusted with trifluoroacetic acid to a pH of 3. The mixture was then extracted three times, each time with 1 litre of ethyl acetate to give a fraction containing M—4', which has an R_f value of 0.46 in thin layer chromatography (Plate: Merck silica gel Art 5715; solvent: a 50:50:3 by volume mixture of

- benzene, acetone and acetic acid). This extract was washed with a saturated aqueous solution of sodium chloride, dried over anhydrous sodium sulphate and subjected to lactonization by adding a catalytic amount of trifluoroacetic acid. The resulting mixture was then washed with a 5% w/v aqueous solution of sodium bicarbonate, dehydrated with anhydrous sodium sulphate and vaporated to dryness.
- 5 The residue was crystallized from ethyl acetate to give about 180 mg. of M—4' lactone having the following physical properties:
- 1) Nuclear Magnetic Resonance Spectrum:
Measured in deuteriochloroform at 100 MHz, using tetramethylsilane as the internal standard.
- 10 δ ppm:
6.01 (1H, doublet);
5.90 (1H, quartet);
5.75 (1H, multiplet);
5.50 (1H, multiplet);
4.60 (1H, multiplet);
15 4.25 (1H, multiplet).
- 2) Ultraviolet Absorption Spectrum (methanol) λ_{\max} nm: 230, 237, 245.
3) Infrared Absorption Spectrum (KBr) ν cm⁻¹: 3500, 1720.
4) Mass spectrum: M/e: (406(M⁺), 304, 286.
5) Optical rotation: $[\alpha]_D^{25} = +310.9^\circ$ (c = 0.66, methanol).
20 6) Melting point: 141—143°C.
7) Elemental analysis:
- Calculated : C, 67.95%; H, 8.43%
Found : C, 68.05%; H, 8.37%.
- 8) Thin Layer Chromatography:
25 TLC plate: Merck silica gel Art 5715.
Solvent: Benzene — acetone (1:1 by volume)
Rf value 0.64.

EXAMPLE 28

Preparation of M—4' sodium salt

- 30 Following substantially the same cultivation procedures as in Example 27, there was obtained a conversion reaction mixture.
- After completion of the cultivation, the conversion reaction mixture was filtered, and the filtrate was adjusted with trifluoroacetic acid to a pH of 3. It was then extracted three times, each time with 1 litre of ethyl acetate to give a fraction containing M—4', which was washed with a saturated aqueous solution of sodium chloride and immediately thereafter passed into a 5% w/v aqueous solution of sodium bicarbonate, to give a fraction containing M—4' sodium salt. The aqueous layer thus obtained
- 35 was adjusted to pH 8.0 with 0.1 N hydrochloric acid and adsorbed on a Diaion HP 20 resin column (manufactured by Mitsubishi Chemical Industries). It was then eluted with 50% v/v aqueous acetone. The acetone was distilled off, and the residue was freeze-dried to give 1.41 g. of M—4' sodium salt,
- 40 having the following physical properties:
- 1) Nuclear Magnetic Resonance Spectrum:
Measured in deuteriochloroform at 60 MHz, using tetramethylsilane as the internal standard.
- 15 δ ppm:
6.00 (1H, doublet);
45 5.95 (1H, quartet);
5.70 (1H, broad singlet);
5.50 (1H, broad singlet).
- 2) Ultraviolet Absorption Spectrum (methanol) λ_{\max} nm: 230, 238, 246.
3) Infrared Absorption Spectrum (KBr) ν cm⁻¹: 3400, 2900, 1680.

50 EXAMPLE 29

Preparation of M—4' methyl ester

- Following substantially the same cultivation procedures as in Example 27, there was obtained a conversion reaction mixture.
- After completion of the cultivation, the conversion mixture was filtered and the filtrate was
- 55 adjusted with trifluoroacetic acid to a pH of 3. It was then extracted three times, each time with 1 litre of ethyl acetate. The combined extracts were washed with a saturated aqueous solution of sodium chloride and then an ethereal solution of diazomethane was added thereto. The resulting mixture was allowed to stand for 30 minutes and then concentrated under reduced pressure to dryness. The residue was purified using a Lobar column (Merck RP—8, size A) and a 1:1 by volume mixture of benzene and
- 60 acetone as the developing solvent. There were obtained 150 mg. of M—4' methyl ester as a colourless

oily substance, having the following properties:

1) Nuclear Magnetic Resonance Spectrum:
Measured in deuteriochloroform at 60 MHz, using tetramethylsilane as the internal standard.

δ ppm:

5 6.01 (1H, doublet);

5.90 (1H, quartet);

5.75 (1H, broad singlet);

5.50 (1H, broad singlet);

3.70 (3H, singlet).

10 2) Ultraviolet Absorption Spectrum (methanol) λ_{\max} nm: 230, 238, 246.

3) Infrared Absorption Spectrum (liquid film) ν cm⁻¹: 3400, 1730.

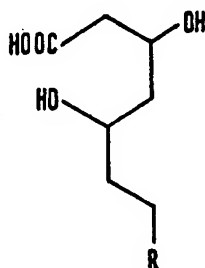
4) Mass analysis:

Measurement was made after silylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide using a mass spectrometer, type D-300 manufactured by Nippon Electronics.

15 M/e: 654 (M⁺).

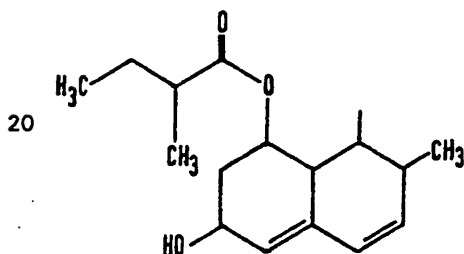
CLAIMS

1. Compounds of formula (I):

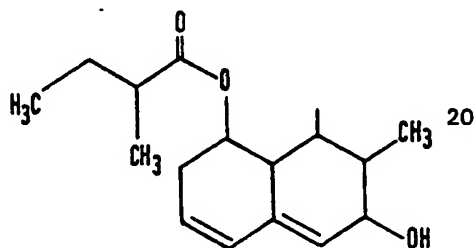


(I)

(in which R represents a group of formula

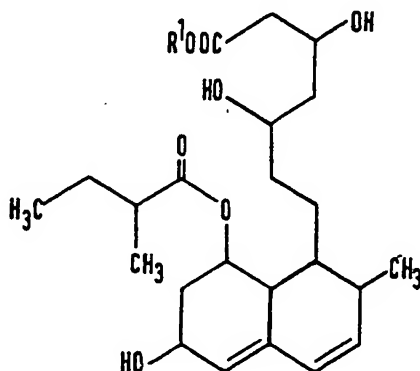


or



and ring-closed lactones, salts and esters thereof.

2. Compounds as claimed in Claim 1, having the formula (II):

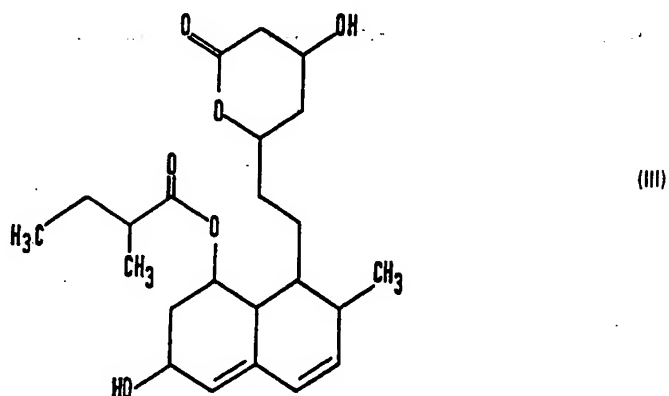


(II)

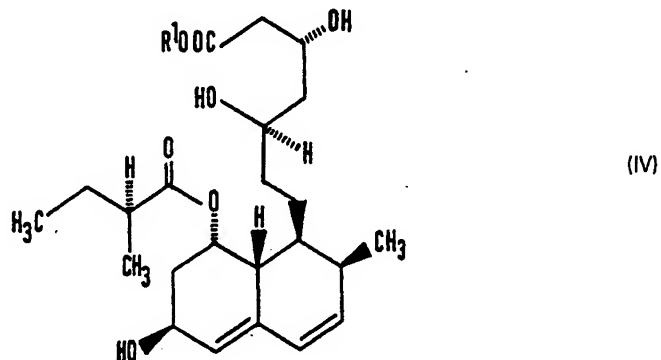
(in which R¹ represents a hydrogen atom or a C₁-C₆ alkyl group) and pharmaceutically acceptable salts
25 of the acid wherein R¹ represents a hydrogen atom.

25

3. A compound as claimed in Claim 1, having the formula (III):



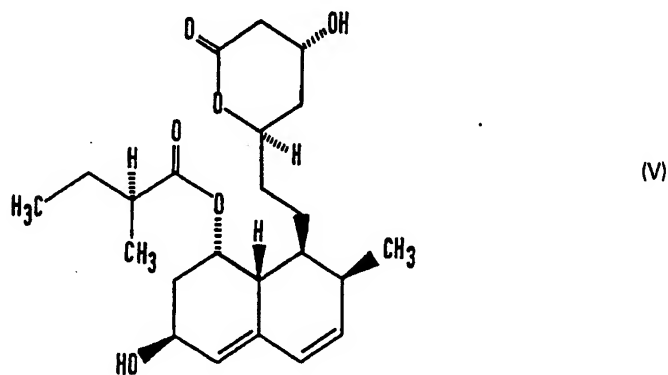
4. Compounds as claimed in Claim 2, having the configuration shown in formula (IV):



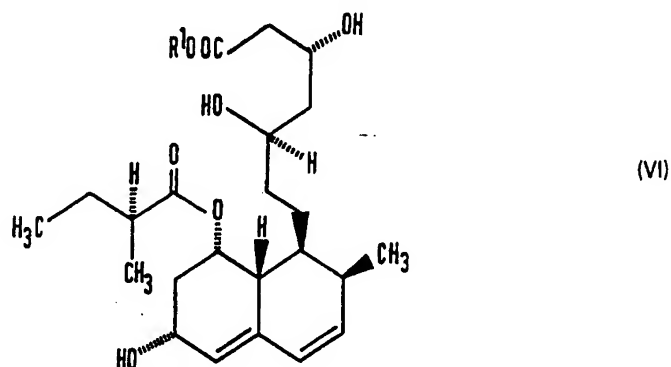
5 (wherein

R^1 is as defined in claim 2), and pharmaceutically acceptable salts thereof.

5. A compound as claimed in Claim 3, having the configuration shown in formula (V):



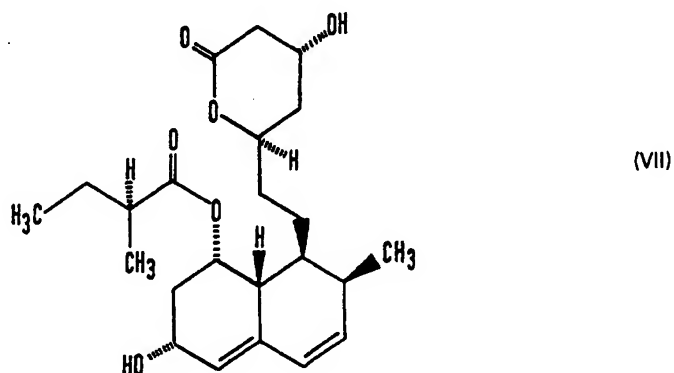
6. Compounds as claimed in Claim 2, having the configuration shown in formula (VI):



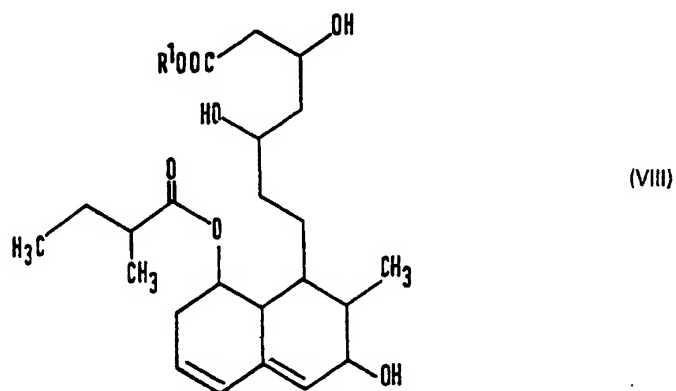
(wherein

R¹ is as defined in Claim 2) and pharmaceutically acceptable salts thereof.

5 7. A compound as claimed in Claim 3, having the configuration shown in formula (VII):



8. Compounds as claimed in Claim 1, which have the formula (VIII):



(in which R¹ represents a hydrogen atom or a C₁—C₈ alkyl group) and pharmaceutically acceptable salts of the acid wherein R¹ represents a hydrogen atom.

10 9. Compounds as claimed in any one of Claims 2, 4, 6 and 8, wherein R¹ represents a hydrogen atom.

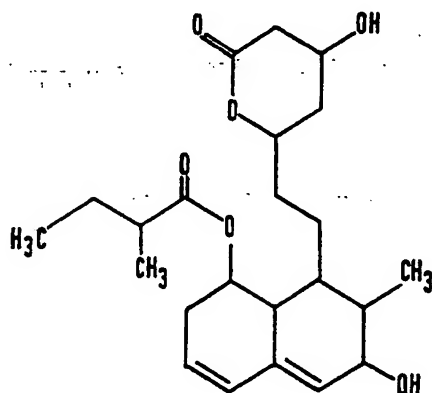
10. Compounds as claimed in any one of Claims 2, 4, 6 and 8, wherein R¹ represents a C₁—C₈ alkyl group.

15 11. Compounds as claimed in any one of Claims 2, 4, 6 and 8, wherein R¹ represents a methyl group.

12. Compounds as claimed in any one of Claims 2, 4, 6 and 8, in the form of the alkali metal salts.

13. Compounds as claimed in Claim 12, in the form of the sodium salt.

14. A compound as claimed in Claim 1, having the formula (IX):



15. A process for preparing a compound as claimed in any one of the preceding claims, which comprises enzymatically hydroxylating ML—236B, or ML—236B carboxylic acid or a salt or ester thereof.

- | | | |
|----|--|----|
| 5 | 16. A process as claimed in Claim 15, wherein the enzymatic hydroxylation is effected by a microorganism of the genus <i>Mucor</i> , <i>Rhizopus</i> , <i>Zygorhynchus</i> , <i>Circinella</i> , <i>Actinomucor</i> , <i>Gongronella</i> , <i>Phycomyces</i> , <i>Martierella</i> , <i>Pycnoporus</i> , <i>Rhizoctonia</i> , <i>Absidia</i> , <i>Cunninghamella</i> , <i>Syncephalastrum</i> or <i>Streptomyces</i> , or with a cell-free, enzyme-containing extract from said microorganisms. | 5 |
| 10 | 17. A process as claimed in Claim 16, wherein said microorganism is: | 10 |
| | <i>Absidia coerulea</i> | |
| | <i>Cunninghamella echinulata</i> | |
| | <i>Syncephalastrum racemosum</i> | |
| | <i>Streptomyces roseochromogenus</i> | |
| | <i>Mucor hiemalis f. hiemalis</i> | |
| 15 | <i>Mucor bacilliformis</i> | 15 |
| | <i>Mucor circinelloides f. circinelloides</i> | |
| | <i>Mucor hiemalis f. corticolus</i> | |
| | <i>Mucor dimorphosporus</i> | |
| | <i>Mucor fragilis</i> | |
| 20 | <i>Mucor genevensis</i> | 20 |
| | <i>Mucor globosus</i> | |
| | <i>Mucor circinelloides f. griseo-cyanus</i> | |
| | <i>Mucor heterosporus</i> | |
| | <i>Mucor spinescens</i> | |
| 25 | <i>Rhizopus chinensis</i> | 25 |
| | <i>Rhizopus circinans</i> | |
| | <i>Rhizopus arrhizus</i> | |
| | <i>Zygorhynchus moelleri</i> | |
| | <i>Circinella muscae</i> | |
| 30 | <i>Circinella rigida</i> | 30 |
| | <i>Circinella umbellata</i> | |
| | <i>Actinomucor elegans</i> | |
| | <i>Phycomyces blakesleeana</i> | |
| | <i>Martierella isabellina</i> | |
| 35 | <i>Gongronella butleri</i> | 35 |
| | <i>Pycnoporus coccineus</i> | |
| | <i>Rhizoctonia solani</i> | |
| | <i>Syncephalastrum nigricans</i> or | |
| | <i>Absidia glauca var. paradoxa</i> . | |
| 40 | 18. A process as claimed in Claim 17, wherein said microorganism is: | 40 |
| | <i>Absidia coerulea</i> IFO—4423 | |
| | <i>Cunninghamella echinulata</i> IFO—4445 | |
| | <i>Cunninghamella echinulata</i> IFO—4444 | |
| | <i>Cunninghamella echinulata</i> ATCC—9244 | |
| 45 | <i>Syncephalastrum racemosum</i> IFO—4814 | 45 |
| | <i>Syncephalastrum racemosum</i> IFO—4828 | |
| | <i>Streptomyces roseochromogenus</i> NRRL—1233 | |
| | <i>Streptomyces roseochromogenus</i> IFO—3363 | |
| | <i>Streptomyces roseochromogenus</i> IFO—3411 | |
| 50 | <i>Mucor hiemalis f. hiemalis</i> IFO—5834 | 50 |

	<i>Mucor hiemalis f. hiemalis</i> IFO—5303	
	<i>Mucor hiemalis f. hiemalis</i> IFO—8567	
	<i>Mucor hiemalis f. hiemalis</i> IFO—8449	
	<i>Mucor hiemalis f. hiemalis</i> IFO—8448	
5	<i>Mucor hiemalis f. hiemalis</i> IFO—8565	5
	<i>Mucor hiemalis f. hiemalis</i> CBS—117.08	
	<i>Mucor hiemalis f. hiemalis</i> CBS—109.19	
	<i>Mucor hiemalis f. hiemalis</i> CBS—200.28	
	<i>Mucor hiemalis f. hiemalis</i> CBS—242.35	
10	<i>Mucor hiemalis f. hiemalis</i> CBS—110.19	10
	<i>Mucor hiemalis f. hiemalis</i> CBS—201.65	
	<i>Mucor bacilliformis</i> NRRL—2346	
	<i>Mucor circinelloides f. circinelloides</i> IFO—4554	
	<i>Mucor circinelloides f. circinelloides</i> IFO—5775	
15	<i>Mucor hiemalis f. corticolus</i> NRRL—12473	15
	<i>Mucor dimorphosporus</i> IFO—4556	
	<i>Mucor fragilis</i> CBS—236.35	
	<i>Mucor genevensis</i> IFO—4585	
	<i>Mucor globosus</i> NRRL—12474	
20	<i>Mucor circinelloides f. griseo-cyanus</i> IFO—4563	20
	<i>Mucor heterosporus</i> NRRL—3154	
	<i>Mucor spinescens</i> IAM—6071	
	<i>Rhizopus chinensis</i> IFO—4772	
	<i>Rhizopus circinans</i> ATCC—1225	
25	<i>Rhizopus arrhizus</i> ATCC—11145	25
	<i>Zygorynchus moelleri</i> IFO—4833	
	<i>Circinella muscae</i> IFO—4457	
	<i>Circinella rigida</i> NRRL—2341	
	<i>Circinella umbellata</i> NRRL—1713	
30	<i>Circinella umbellata</i> IFO—4452	30
	<i>Circinella umbellata</i> IFO—5842	
	<i>Phycomyces blakesleeana</i> NRRL—12475	
	<i>Martierella isabellina</i> IFO—6739	
	<i>Gongronella butleri</i> IFO—8080	
35	<i>Pycnopus coccineus</i> NRRL—12476	35
	<i>Rhizoctonia solani</i> NRRL—12477	
	<i>Syncephalastrum nigricans</i> NRRL—12478	
	<i>Syncephalastrum nigricans</i> NRRL—12479	
	<i>Syncephalastrum nigricans</i> NRRL—12480	
40	<i>Absidia glauca</i> var. <i>paradoxa</i> IFO—4431 or	40
	<i>Actinomucor elegans</i> ATCC—6476	
	19. A process as claimed in any one of Claims 15 to 18, wherein there is separated from the reaction mixture one or more of M—4, M—4', IsoM—4, IsoM—4' or a salt, ester or lactone of M—4, M—4' IsoM—4 or IsoM—4'.	
45	20. A process as claimed in Claim 19, wherein said ester is a C ₁ —C ₈ alkyl ester.	45
	21. A process as claimed in Claim 19, wherein said ester is the methyl ester.	
	22. A process as claimed in Claim 19, wherein said salt is an alkali metal salt.	
	23. A process as claimed in Claim 19, wherein said salt is a sodium salt.	
	24. A process as claimed in Claim 15, wherein said microorganism is:	
50	<i>Absidia coerulea</i>	50
	<i>Cunninghamella echinulata</i>	
	<i>Syncephalastrum racemosum</i>	
	<i>Mucor hiemalis f. hiemalis</i>	
	<i>Mucor bacilliformis</i>	
55	<i>Mucor circinelloides f. circinelloides</i>	55
	<i>Mucor hiemalis f. corticolus</i>	
	<i>Mucor dimorphosporus</i>	
	<i>Mucor fragilis</i>	
	<i>Mucor genevensis</i>	
60	<i>Mucor globosus</i>	60
	<i>Mucor circinelloides f. griseo-cyanus</i>	
	<i>Mucor heterosporus</i>	
	<i>Mucor spinescens</i>	
	<i>Pycnopus coccineus</i>	
65	<i>Rhizoctonia solani</i>	65

Syncephalastrum nigricans.

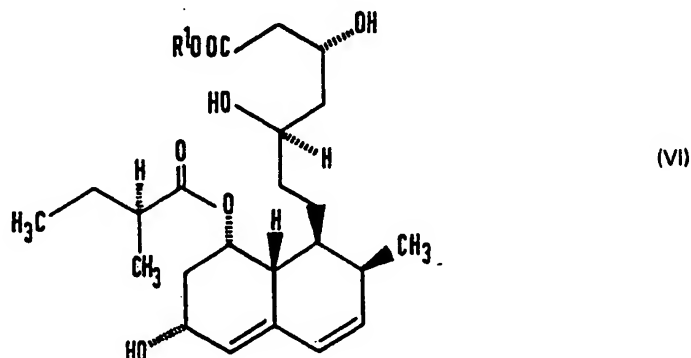
25. A process as claimed in Claim 15, wherein said microorganism is:

- | | | |
|----|--|----|
| | <i>Absidia coerules</i> IFO—4423 | |
| | <i>Cunninghamella echinulata</i> IFO—4445 | |
| 5 | <i>Cunninghamella echinulata</i> IFO—4444 | 5 |
| | <i>Cunninghamella echinulata</i> ATCC—9244 | |
| | <i>Syncephalastrum racemosum</i> IFO—4814 | |
| | <i>Syncephalastrum racemosum</i> IFO—4828 | |
| | <i>Mucor hiemalis f. hiemalis</i> IFO—5834 | |
| 10 | <i>Mucor hiemalis f. hiemalis</i> IFO—5303 | 10 |
| | <i>Mucor hiemalis f. hiemalis</i> IFO—5304 | |
| | <i>Mucor hiemalis f. hiemalis</i> IFO—8567 | |
| | <i>Mucor hiemalis f. hiemalis</i> IFO—8449 | |
| | <i>Mucor hiemalis f. hiemalis</i> IFO—8448 | |
| 15 | <i>Mucor hiemalis f. hiemalis</i> IFO—8565 | 15 |
| | <i>Mucor hiemalis f. hiemalis</i> CBS—117.08 | |
| | <i>Mucor hiemalis f. hiemalis</i> CBS—109.19 | |
| | <i>Mucor hiemalis f. hiemalis</i> CBS—200.28 | |
| | <i>Mucor hiemalis f. hiemalis</i> CBS—242.35 | |
| 20 | <i>Mucor hiemalis f. hiemalis</i> CBS—110.19 | 20 |
| | <i>Mucor hiemalis f. hiemalis</i> CBS—201.65 | |
| | <i>Mucor bacilliformis</i> NRRL—2346 | |
| | <i>Mucor circinelloides f. circinelloides</i> IFO—4554 | |
| | <i>Mucor circinelloides f. circinelloides</i> IFO—5775 | |
| 25 | <i>Mucor hiemalis f. corticolus</i> NRRL—12473 | 25 |
| | <i>Mucor dimorphosporus</i> IFO—4556 | |
| | <i>Mucor fragilis</i> CBS—236.35 | |
| | <i>Mucor genevensis</i> IFO—4585 | |
| | <i>Mucor globosus</i> NRRL—12474 | |
| 30 | <i>Mucor circinelloides f. griseo-cyanus</i> IFO—4563 | 30 |
| | <i>Mucor heterosporus</i> NRRL—3154 | |
| | <i>Mucor spinescens</i> IAM—6071 | |
| | <i>Pycnoporus coccineus</i> NRRL—12476 | |
| | <i>Rhizoctonia solani</i> NRRL—12477 | |
| 35 | <i>Syncephalastrum nigricans</i> NRRL—12478 | 35 |
| | <i>Syncephalastrum nigricans</i> NRRL—12479 or | |
| | <i>Syncephalastrum nigricans</i> NRRL—12480. | |

26. A process as claimed in Claim 15, wherein said microorganism is:

- | | | |
|----|---|----|
| | <i>Mucor hiemalis f. hiemalis</i> | |
| 40 | <i>Mucor circinelloides f. circinelloides</i> | 40 |
| | <i>Mucor fragilis</i> | |
| | <i>Mucor genevensis</i> | |
| | <i>Mucor circinelloides f. griseo-cyanus</i> | |
| | <i>Pycnoporus coccineus</i> or | |
| 45 | <i>Rhizoctonia solani</i> . | 45 |

27. A process as claimed in Claim 15, wherein there is prepared a compound of formula (VI):



(wherein

R¹ represents a hydrogen atom or a C₁—C₈ alkyl group), a pharmaceutically acceptable salt of the
 50 acid wherein R¹ represents a hydrogen atom, or a compound of formula (VII):

50